ISSN 1364-7565 Acta Phytotax. Geobot. 55 (1): 9–18 (2004)

Molecular Systematic Study of Asian *Conocephalum japonicum* (Hepaticae)

HIDETSUGU MIWA[1], TSAI-WEN HSU[2], XIAO CHENG[3], JUMPEI SUHARA[4] and NORIAKI MURAKAMI[1]

[1]Department of Botany, Graduate School of Science, Kyoto University, Kitashirakawa-Oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan; [2]Division of Botany, Taiwan Endemic Species Research Institute, 1, Ming-Shen E. Road, Chi-Chi, Nan-Tou 552, Taiwan; [3]Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan 650204, China; [4]Department of Life Science, Faculty of Science, Rikkyo (St.Paul's) University, 3-34-1, Nishi-ike-bukuro, Toshima-ku, Tokyo 171-8501, Japan

In our previous study, we found two *rbcL* types (JN and JS types) in Japanese *Conocephalum japonicum* Grolle. In this study, we collected plant materials from 20 localities in China, Korea and Taiwan. We determined 1304 bps of each nucleotide *rbcL* sequence and found two *rbcL* types in Asian *C. japonicum.* One of them was a new *rbcL* type (CS type), which differs from the JN and JS types by 10 and 8 nucleotides, respectively. The CS and JS types were found both in Mainland China and Taiwan. We could not find any morphological differences between the two types. In Chitou, Taiwan, the two *rbcL* types were growing together within a small research area measuring 100 m x 200 m. We performed an allozyme analysis on this population and observed that the CS type had *Est-4a* and *Tpi-1a* alleles; whereas the JS type had *Est-4b* and *Tpi-1b*. It was strongly suggested that these two *rbcL* types might be reproductively isolated cryptic species of *C. japonicum.*

Key words: allozyme, bryophytes, *Conocephalum japonicum,* cryptic species, genetic variation, *rbcL*, reproductive isolation

Bryophytes are typically the smallest land plants having a very simple morphology. However, most species of bryophytes have been recognized and described mostly by their limited morphological information. Morphological species of bryophytes often have a broad geographical distribution spanning two or more continents (Schofield & Crum 1972). Even though bryophytes can be easily dispersed to remote sites through minute spores, such ranges seem too wide for the disrtribution of a single biological species. Therefore, we can expect that several cryptic species, which are genetically and ecologically well-differentiated from each other but morphologically hardly distinguished, might be contained within a single morphological species. In fact, cryptic speciation has been demonstrated in some broadly distributed bryophyte species such as the thallose liverworts (*Conocephalum conicum* Dumort., *Marchantia polymorpha* L., *Reboulia hemisphaerica* Raddi, *Riccia dictyospora* Howe, *Aneura pinguis* Dumort., *Pellia epiphylla* Corda), and a leafy liverwort (*Porella platyphylla* Pfeiff) as well as several mosses (*Climacium americanum* Brid., *Leucobryum glaucum* Angstr., *Plagiomunium*

cuspidatum T.J.Kop., *Mielichhoferia elongata* Nees & Hornsch. and *Fontinalis antipyretica* Hedw). (reviewed by Shaw 2001). These cryptic species have mainly been determined through allozyme analysis.

Among these examples, the first known case of cryptic speciation in bryophtyes was demonstrated using the thallose liverwort *Conocephalum conicum*. Odrzykoski & Szweykowski (1991), Akiyama & Hiraoka (1994) and Kim *et al.* (1996) examined allozyme variation from throughout the geographical distribution range of the species, and found six discrete groups in total. The genetic differentiation among these six groups as measured by Nei's genetic distance was as large as those commonly reported between different vascular plant species and much larger than those between conspecific populations of a higher plant species. They found several mixed populations of two different allozyme types of *C. conicum*, but even in these sympatrical populations, recombinant gametophytes were not detected (Szweykowski *et al.* 1981, Odrzykoski 1987). They concluded that these six allozyme groups were biologically independent cryptic species.

In our previous study (Miwa *et al.* 2003), we sought to find cryptic species in *Conocephalum japonicum* Grolle using *rbcL* sequence variations rather than allozyme analysis as a preliminary indicator. In angiosperms, *rbcL* variations have been considered to be useful only for inter-generic or inter-familial level of phylogenetic analyses (Chase *et al.* 1993). In various ferns, however, *rbcL* variation has been shown to be a useful tool for finding cryptic species (Murakami *et al.* 1998a, b, Yatabe *et al.* 1998, 1999, 2001, Kato *et al.* 2001, Masuyama *et al.* 2002). We considered it highly possible that *rbcL* variation would also be useful for finding cryptic species of bryophytes.

Actually in our previous study (Miwa *et al.* 2003), two distinct types of *rbcL* sequences (JN and JS), differing by 6 nucleotides, were found in Japanese *Conocephalum japonicum*. Their geographical distribution areas in Japan were clearly separated by the band of latitude 42-43°N through Hokkaido. The geographical boundary of the two *rbcL* types is nearly identical to the northern limit of the distribution range of *Fagus crenata* in Japan (Horikawa 1972). We found a mixed population of JN and JS types in Abuta, Hokkaido, Japan. At this site, the two *rbcL* types were growing side by side in 2-3 meters distance. We also conducted allozyme analysis and detected polymorphisms in EST and TPI enzymes, and tight associations between the allozyme and *rbcL* variants were observed; the JS type were fixed for *Est-3a* and *Tpi-1a* alleles, whereas the JN type for *Est-3b* and *Tpi-1b*. Individuals with genotypes of other combinations were not found in this population. Thus, we tentatively concluded that these two *rbcL* types of *C. japonicum* represented two reproductively isolated cryptic species.

Conocephalum japonicum is widely distributed in East Asia: Nepal, Japan, Kamchatka (Russia) and Taiwan mark the west, east, north, and south boundaries of its range (Kitagawa 1982). In the present study, we collected samples of *C. japonicum* in China, Korea and Taiwan in order to discover possible new *rbcL* types and also to elucidate the whole distribution range of the two *rbcL* types found in Japan.

Materials and Methods

Plant materials

We collected *Conocephalum japonicum* at 20 remote localities in China, Korea and Taiwan (Table 1). We generally collected a single individual from each locality. For one population at Chitou, Taiwan, we mapped all the recognizable individuals growing in a 100 m x 200 m transect, and then collected a small amount of each of 21 plants for *rbcL* sequencing and enzyme electrophoretic analysis. All voucher specimens are deposited in the Herbarium of the

Graduate School of Science, Kyoto University (KYO).

DNA sequencing and molecular phylogenetic analysis

Total DNAs were extracted using the Nucleon PhytePure plant and fungal DNA extraction kit (Amersham), or alternatively the Plant DNeasy Kit (Qiagen). Partial *rbcL* gene segments were amplified by PCR using Ready-To-Go PCR Beads (Amersham). We used two sets of primers: 1-1 (ATGTCACCACAAACAGAGACTAAAGC), 2R (CTTCTGCTACAAATAAGAATCGATCTCTCCA), N2-1 (TGAAAACGTGAATTCCCAACCGTTTATGCG), NN3-2 (GCAGCAGCTAGTTCCGGGCTCCA) for the PCR (Hasebe *et al.* 1994). A typical PCR amplification included an initial denature (5 min, 94℃) followed by 35 cycles with a 1 min denature at 94℃, 1 min annealing at 50-55℃, 2 min 30 sec synthesis at 72℃, and a final step of synthesis for 6 min at 72℃. The PCR products were purified using a QIA quick PCR Purification Kit (Qiagen). The amplified fragments were sequenced with a Big Dye terminator cycle sequencing kit (Applied Biosystems) using the above mentioned primers, and run on an Applied Biosystems Model 377 automated DNA sequencer (Applied Biosystems). The obtained sequences were handled by Sequence Navigator software (Applied Biosystems). A molecular phylogenetic tree was

TABLE 1. Voucher information of the plant materials from China, Korea and Taiwan. Abbreviation of collectors: XC, X. Cheng; TH, T.-W. Hsu; HM, H. Miwa; JS, J. Suhara.

Locality	Collectors	Specimen No.
C. japonicum (CS type)		
1. Kunming, Yunnan, China [2000 m]	*JS and XC*	*HM4002*
2. Wuding, Yunnan, China [2000 m]	*JS and XC*	*HM1272*
3. Daguan, Yunnan, China [1300 m]	*JS and XC*	*HM3305*
4. Shilin, Yunnan, China [1500 m]	*JS and XC*	*HM4014*
5. Malipo, Yunnan, China [750 m]	*JS and XC*	*HM4061*
6. Xichou, Yunnan, China [1500 m]	*JS and XC*	*HM4062*
7. Hekou, Yunnan, China [1150 m]	*JS and XC*	*HM4006*
8. Taipingshan, Ilan, Taiwan [890 m]	*HM and TH*	*HM3351*
9. Taipingshan, Ilan, Taiwan [1840 m]	*HM and TH*	*HM3356*
C. japonicum (CS/JS type)		
10. Chitou, Nantou, Taiwan [ca.1150 m]	*HM and TH*	*HM3209C1-21*
C. japonicum (JS type)		
11. Kochiu, Yunnan, China [1490 m]	*JS and XC*	*HM4013*
12. Manhaozhen, Yunnan, China [540 m]	*JS and XC*	*HM4010*
13. Adebo, Yunnan, China [1335 m]	*JS and XC*	*HM4011*
14. Jinpin, Yunnan, China [1880 m]	*JS and XC*	*HM4012*
15. Pinbian, Yunnan, China [1250 m]	*JS and XC*	*HM4005*
16. Tianwangmiao, Taipei, Taiwan [470 m]	*HM and TH*	*HM3361*
17. Mucha, Taipei, Taiwan [300 m]	*HM and TH*	*HM3391*
18. FuanHuag, Nantou, Taiwan [865 m]	*HM and TH*	*HM3381*
19. Cheju, Cheju-isl., Korea [600 m]	*HM*	*HM3111*
20. Cheju, Cheju-isl., Korea [720 m]	*HM*	*HM3113*

constructed using the CLUSTALX program, version 1.8 (Thompson *et al.* 1997).

Allozyme analysis

About 100 mg of fresh thallus tissue was homogenized in a 1 ml extraction buffer (0.2% 2-mercaptoethanol, 2% polyvinyl-pyrrolidone, 0.1 mM Tris-HCl, 1 mM EDTA4Na, 10 mM KCl, 10 mM MgCl2, pH = 7.5). After centrifuging the homogenates at 10,000 rpm for 10 minutes at 4℃, 20 μl of the supernatant was used for electrophoresis for detecting each enzyme. Vertical polyacrylamide slab gel electrophoresis using a discontinuous buffer system (Shiraishi 1988) was conducted for genotyping all plant samples collected from the Chitou population. Sixteen enzyme systems were tested for activity, and scorable bands were obtained for the following 10 enzymes: fluorescent esterase (EST E.C.: 3.1.1.1), triosephosphate isomerase (TPI E.C.:5.3.1.1), 6-phosphoglconate dehydrogenase (6PG E.C.:1.1.1.44), shikimate dehydrogenase (SKD E.C.:1.1.1.25), glutamate dehydrogenase (GDH E.C.:1.4.1.2), diaphorase (DIA E.C.:1.6.4.3), phosphoglucoisomerase (PGI E.C.:5.3.1.9), glutamate oxaloacetate transaminase (GOT E.C.:2.6.1.1), acid phosphatase (ACP E.C.:3.1.3.2), and superoxidedesmutase (SOD E.C.:1.15.1.1). The loci were numbered in relation to their mobility: the longest distance zone was isozyme *1*, and shorter distance zones were isozymes *2*, *3* and so on. The allele labels alphabetically denote relative mobility, the *a*-alleles of each locus have the longest distance, followed by *b*, *c* and so on.

Results

We determined 1,304 nucleotide sequences of the *rbcL* gene for all the collected samples of *Conocephalum japonicum*. Two distinct *rbcL* sequences were found in Asian *C. japonicum* (Fig. 1). One of them was a new *rbcL* type, which differs from JN and JS types by 10 and 8 nucleotides, respectively. We named the new *rbcL* type as CS (China South) type. The CS type was found in the northern part of Yunnan-province, China and at higher elevations of Taiwan. The JS type was found in Korea, Yunnan-province, China and Taiwan. The geographical distribution of CS and JS types is shown in Fig. 2, along with the distribution data of the JS and JN types in our previous work (Miwa *et al.* 2003). The JN type was not found among the samples in Mainland China and Taiwan.

In Chitou, Taiwan, we found a mixed population of JS and CS types (16 individuals of JS type and 5 individuals of CS type). In this site, the two *rbcL* types were growing side by side within a small research area measuring 100 m x 200 m (Fig. 3). We performed an allozyme analysis of the 21 samples collected from the Chitou population. Polymorphisms were detected only in EST and TPI. In addition, the JS type always had *Est-4b* and *Tpi-1b* alleles, whereas the CS type always had *Est-4a* and *Tpi-1a*, and thus tight associations between allozyme and *rbcL* variations were observed (Fig. 4). The appearances of the three *rbcL* types are shown in Fig. 5. Following the study on *Conocephalum conicum* by Odrzykoski & Szweykowski (1991) and Akiyama & Hiraoka (1994), we observed morphological features of cells and air chambers. We also compared length, width, thickness of thalli as well as their color among JN, JS and CS types. We could not find any significant morphological differences among the three *rbcL* types.

We made a molecular phylogenetic analysis based on the obtained *rbcL* sequences of *Conocephalum japonicum* together with those of other bryophytes including *C. conicum* from the DNA database (Fig. 6). The *rbcL* sequences of *C. japonicum* and *C. conicum* made a separate clade on the obtained molecular tree. Among the three *rbcL* types of *C. japonicum*, the JS and CS types were shown to be more closely related to each other than either is to the JN type.

```
JN) ---------- ---------- ---------- ---GGATTCA AAGCTGGTGT TAAAGATTAT AGATTAACTT ATTACACTCC   80
JS)                                                                                     C
CS)                                                                                     A

JN) GGATTATGAA ACTAAAGAGA CAGATATTTT AGCAGCATTT AGAATGACTC CTCAGCCTGG GGTACCAGCA GAAGAAGCAG  160
JS)                    T                                                         C
CS)                    T                                                         G

JN) GAGCAGCAGT TGCTGCTGAA TCTTCTACCG GTACATGGAC TACAGTTTGG ACTGATGGTC TTACTAATCT TGATCGTTAT  240
JS)
CS)

JN) AAAGGTCGAT GCTATGGTAT TGACCCTGTT GCTGGAGAAG AAAATCAATA TATTGCTTAT GTAGCTTATC CTTTAGATTT  320
JS)                                  C
CS)                                  C

JN) ATTTGAAGAA GGATCTGTTA CAAATATGTT TACTTCTATT GTAGGTAATG TATTTGGATT TAAAGCTTTA AGAGCATTAC  400
JS)
CS)

JN) GTCTTGAAGA TTTACGAATT CCTCCAGCTT ATACAAAAAC TTTCCAAGGT CCTCCTCATG GTATTCAAGT TGAAAGAGAT  480
JS)
CS)

JN) AAATTAAACA AATATGGTCG TCCTTTATTA GGATGTACTA TTAAACCAAA ATTAGGTTTA TCTGCTAAAA ATTATGGTAG  560
JS)
CS)

JN) AGCTGTATAT GAATGTCTTC GTGGTGGACT TGACTTTACT AAAGATGATG AAAATGTAAA TTCTCAACCA TTTATGCGTT  640
JS)
CS)

JN) GGAGAGATCG TTTCTTATTT GTAGCAGAAG CTCTTTATAA ATCTCAATCA GAAACTGGAG AAATCAAAGG ACATTATTTA  720
JS)                                        T           T
CS)                                        T           G

JN) AATGCTACTG CAGGTACATC TGAAGAAATG TTAAAAAGAG CAGCATGTGC TAGAGAGTTA GGTGTACCAA TTGTTATGCA  800
JS)
CS)

JN) TGACTATTTA ACTGGTGGTT TTACTGCAAA TACTAGTTTA TCTCATTATT GCCGTGATAA TGGTTTACTT CTTCATATTC  880
JS)                            T
CS)                            T

JN) ACCGTGCAAT GCATGCAGTT ATTGATAGAC AAAAAAATCA TGGTATCCAT TTCCGTGTAT TAGCTAAAGC TTTACGTTTA  960
JS)
CS)

JN) TCTGGTGGAG ACCATATTCA TGCTGGTACT GTTGTAGGTA AACTTGAAGG AGACCGTCAA GTTACTTTAG GTTTCGTAGA 1040
JS)                                                               G                  C
CS)                                                               C                  T

JN) TTTACTTCGT GATGATTATA TCGAAAAAGA TAGAAGTCGT GGTATTTATT TTACACAAGA TTGGGTTTCT TTACCAGGTG 1120
JS)
CS)

JN) TTCTACCTGT AGCGTCTGGA GGAATTCATG TTTGGCATAT GCCTGCTTTA ACTGAAATTT TTGGAGATGA CTCTGTTTTA 1200
JS)                                                                              C
CS)                                                                              T

JN) CAATTTGGTG GTGGAACTTT AGGCCATCCT TGGGGTAACG CACCTGGTGC AGTTGCTAAC CGAGTTGCTT TAGAAGCGTG 1280
JS)                          C               C
CS)                          T               T

JN) TGTACAAGCA CGTAATGAAG GTCGTGATCT TGCTCGTCJNA GGTAATGAAA TTATTCGTGA ---------- --------- 1360
JS)
CS)
```

FIG. 1. Nucleotide sequences of the *rbcL* gene of three types in Asian *Conocephalum japonicum*. The variable sites are enclosed in boxes. The sequence data have been deposited in the DDBJ DNA database with the accession number JN type: AB046694, JS type: AB046695 and CS type: AB046719.

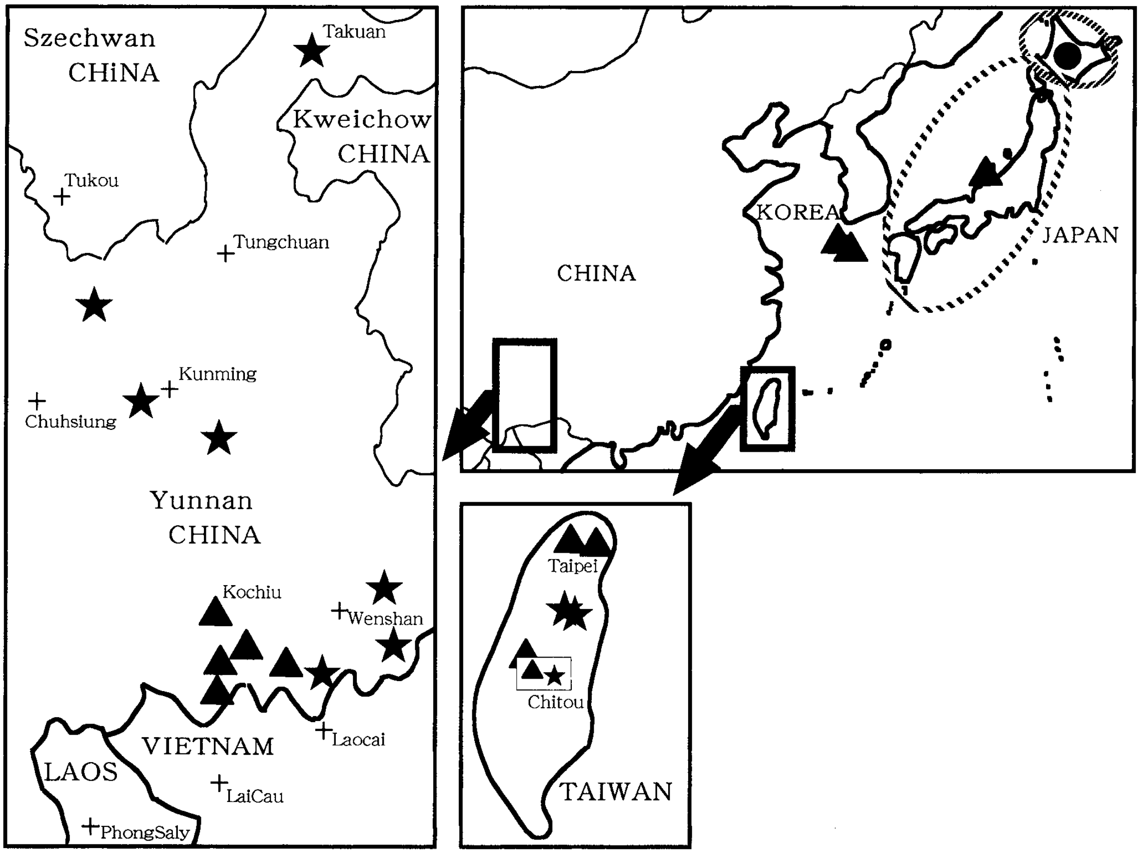

FIG. 2. The geographical distribution of the CS type (★), JS type (▲) and JN type (●) of *Conocephalum japonicum* in Asia. Details for Japanese populations are shown in Miwa *et al.* (2003).

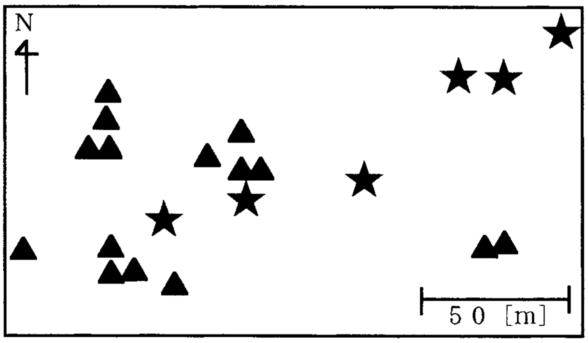

FIG. 3. Map showing the distribution of the CS (★) and JS type (▲), of *Conocephalum japonicum* in the Chitou population, Taiwan.

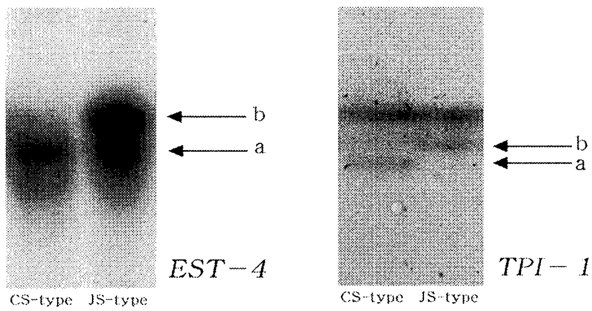

FIG. 4. The allozyme phenograms (EST and TPI) of the CS and JS types of *Conocephalum japonicum* in the Chitou population, Taiwan.

Discussion

The 8-10 substitutions among the three *rbcL* types of *Conocephalum japonicum* may represent a large genetic distance considering the slow evolutionary rate of the *rbcL* gene of higher plants (Chase *et al.* 1993, Yatabe *et al.* 1999). In order to test the hypothesis that these three *rbcL* types are reproductively isolated cryptic species, it is important to examine a sympatrical population where the two or more *rbcL* types were found growing together, as we did for the JN and JS types using their mixed

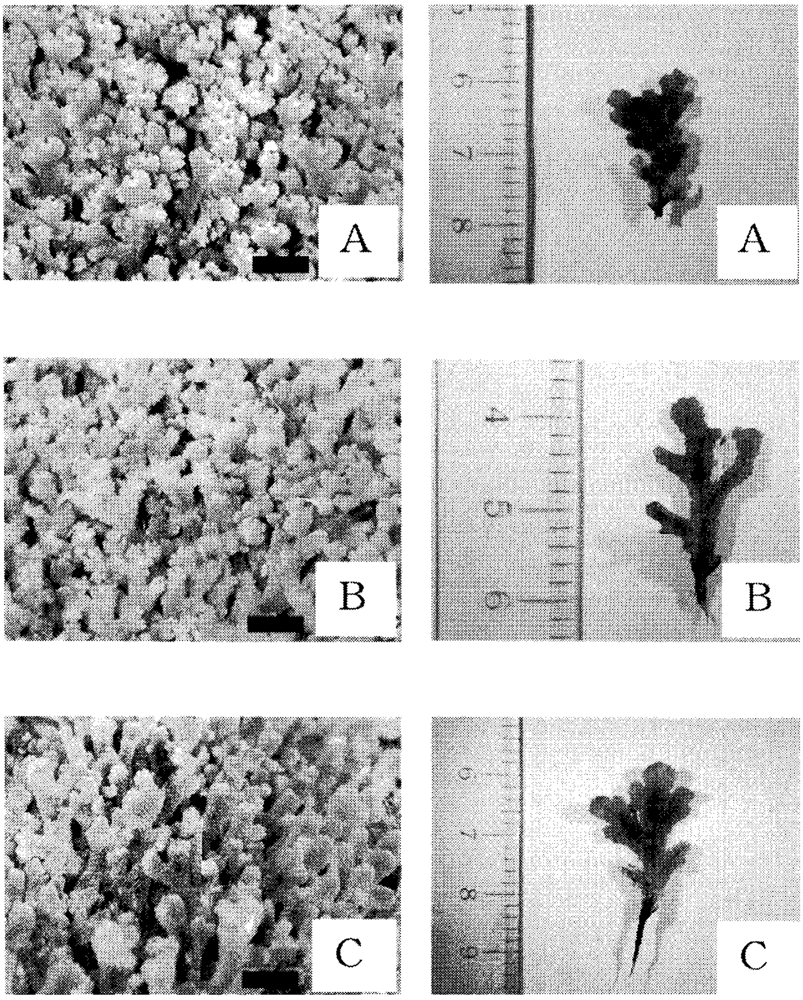

FIG. 5. General appearances of *Conocephalum japonicum*. A, JN type from Hokkaido, Japan. B, JS type from Kanagawa, Japan. C, CS type from Ilan, Taiwan. Bar, 1 cm.

population in Abuta, Hokkaido, Japan (Miwa *et al.* 2003). In this study, the allozyme analysis for the Chitou population showed that the JS and CS types, both recognized by chloroplast gene differentiation, were genetically fixed to different alleles in the *Est-4* and *Tpi-1* loci. Thus, genetic differentiation in two nuclear genes between the two *rbcL* types was clearly shown in this study as in the case of JN and JS types. Their results suggested that the three *rbcL* types (JN, JS and CS) are different biological species, because the results based on two independent gene markers, cpDNA (*rbcL*) and allozyme (nuclear), showed strong correlation.

At this moment, we can not exclude the possibility that all individuals of the Abuta and Chitou populations might be interfertile because *Conocephalum japonicum* produces numerous gemmae, and can propagate by an asexual process as well. We need to perform artificial crossing experiments in order to clearly show the existence of reproductive isolation among the three *rbcL* types. Anyway, we have found three genetically well-differentiated *rbcL* types in *C. japonicum*.

Shaw (2001) noted that most of the cryptic species documented within widespread liverworts are genetically monomorphic or nearly so, in spite of

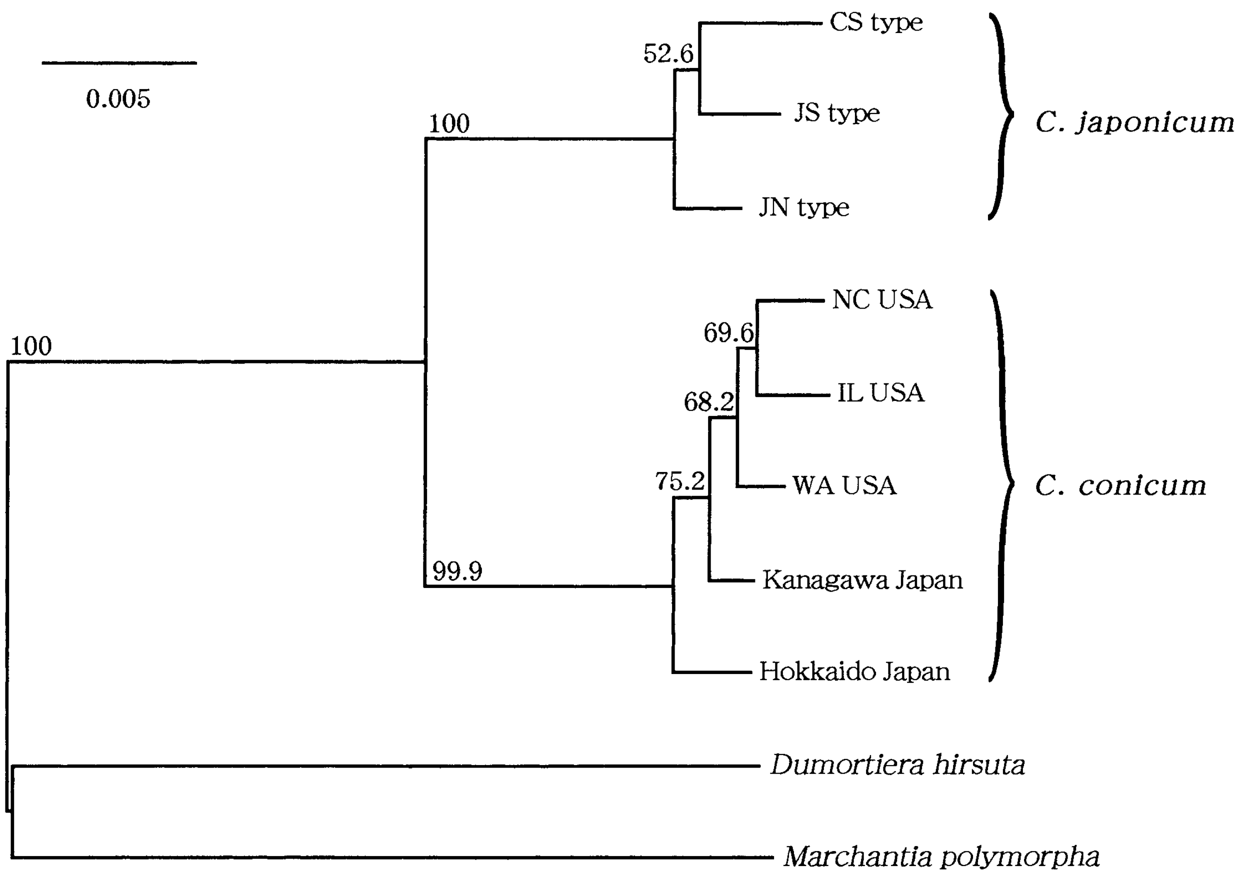

FIG. 6. Neighbor-joining tree based on the nucleotide sequences of the *rbcL* gene. *Marchantia polymorpha* (DNA database accession No X04465) and *Dumortiera hirsuta* (No U87068) were used as outgroups. We also added samples of *Conocephalum conicum* from five distant localities: WA, U.S.A. (No AB056154), Kanagawa, Japan (No AB046697), Hokkaido, Japan (No AB046696), NC, U.S.A. (No U87066), IL, U.S.A. (No U87067) for comparison. Numbers above the branches are the bootstrap values (%). Bootstrap analyses were accomplished using 1000 bootstrap replicates.

high levels of genetic difference among them. He discussed how the origin of these cryptic species might be related to highly inbred or clonal reproduction. The genetic situation found in *Conocephalum japonicum* is in fact the same as those generally found in other widespread liverworts. Capacity for clonal reproduction of *C. japonicum* might be related to its cryptic speciation.

This study also suggested that *rbcL* sequence variation is useful in searching for cryptic species of bryophytes. Many cryptic species of bryophytes have been found using allozyme analyses (see Shaw 2001). However, cryptic species-specific alleles were rarely found, and it is usual that allozyme variations are observed in each cryptic species. To date, cryptic species recognized by allozyme analyses have generally been identified as clusters in UPGMA dendrograms based on Nei's genetic distances (Dewey 1989, Odrzykoski & Szweykowski 1991, Akiyama & Hiraoka 1994). This indicates that bryophyte cryptic species were first recognized by differentiation in allele frequencies of several independent allozyme loci. Cryptic species-specific allozyme bands can always be found and identified after clusters of their population have been recognized. In other words, allozyme analyses usually help in identifying only populations, even when the analysis is a search for cryptic species. Therefore, if plants of different cryptic species are growing sympatrically, as in the case of JS and CS

types in the Chitou population, it may be difficult to find cryptic species using allozyme analyses.

In such cases, we expect *rbcL* sequence analysis to be useful because it is always applicable on the individual level. The *rbcL* genes are encoded by chloroplast DNA, which is usually treated as maternally inheriting without recombination. Maternal inheritance of the plastid genome was also documented in one species of bryophytes (*Anthoceros punctatus* L.) by Izumi & Ono (1999). In this case, effective population size for maternally inherited genes is expected to be one quarter of that for the biparentally inherited nuclear genes such as allozyme loci (Moore 1995). Therefore, genes encoded by cpDNA are more easily fixed in each Mendel population by random genetic drift. This means that their variations, if available, tend to coincide with the boundaries of other biological species even in mixed populations.

Moreover, samplings are much easier in *rbcL* sequencing than in allozyme analysis, because even silica gel dried samples are sufficient for the former method, whereas fresh materials are really necessary for the latter. For allozyme analyses, standard plant samples are also necessary for identifying allozyme bands in different electrophoresis, but it is difficult and laborious to keep bryophyte samples alive for long periods. Such procedures are completely unnecessary for *rbcL* analyses.

These advantages of cpDNA sequence data suggest *rbcL* sequences to be more useful than allozyme data as a preliminary indicator of reproductively isolated assemblages or biological species (Yatabe *et al.* 2002). In fact, *rbcL* variations have been shown to be a good key for finding cryptic species in various fern groups. The present study suggests that *rbcL* variations are also useful for identifying cryptic species of bryophytes. We consider it necessary to examine actual circumstances of *rbcL* variation among the cryptic species recognized by allozyme analyses in previous cases. In addition, usefulness of *rbcL* variation in searching for cryptic species will be also elucidaced if we find a new one in *Conocephalum conicum*, in which cryptic species have been well sought by allozyme analyses. It is indeed, our next step to establish the new, more efficient method for recognizing biological species in bryophtytes.

The authors thank Dr. A. Seo, Kyoto Univ., Dr. R. Hanai, Rikkyo (St.Paul's) Univ. and Dr. S.-J. Moore, National Taiwan Normal Univ. for assistance in plant collection. The authers also thank Dr. N. Kitagawa, Nara Sangyo Univ. for discussions on bryophytes. This study was partly supported by a grant from the New Technology Development Foundation to JS, Grants-in-Aid from Japan Society for the Promotion of Science No. 13575012 and 15370037 to NM, and Grant-in-Aid of Frontier Project from the Ministry of Education, Science, Sports and Culture, Japan to Rikkyo (St.Paul's) University.

References

Akiyama, H. & T. Hiraoka. 1994. Allozyme variability within and divergence among populations of the liverwort *Conocephalum conicum* (Marchantiales: Hepaticae) in Japan. J. Plant Res. 107: 307-320.

Chase, M. W., D. E. Soltis, R. G. Olmstead, D. Morgan, D. H. Les, B. D. Mishler, M. R. Duvall, R. A. Price, H. G. Hills, Y. Qiu, K. A. Kron, J. H. Retting, E. Conti, J. D. Palmer, J. R. Manhart, K. J. Sytsma, H. J. Michaels, W. D. Clark, M. Hedren, B. S. Gaut, R. K. Jansen, K. Kim, C. F. Wimpee, J. F. Smith, G. R. Furnier, S. H. Straus, Q. Xiang, G. M. Swensen, S. E. Williams, P. A. Gadek, C. J. Quinn, L. E. Eguiarte, E. Golenberg, G. H. Learn, S. W. Jr. Graham, S. C. H. Barret, S. Dayanandan & V. A. Albert. 1993. Phylogenetics of seed plants: an analysis of nucleotide sequences from the plastid gene *rbcL*. Ann. MO Bot. Gard. 80: 528-580.

Dewey, M. R. 1989. Genetic variation in liverwort *Riccia dictyospora* (Ricciaceae, Hepaticopsida). Syst. Bot. 14: 155-167.

Hasebe, M., T. Omori, M. Nakazawa, T. Sano, M. Kato & K. Iwatsuki. 1994. *RbcL* gene sequences provide evidence for the evolutionary lineages of leptosporangiate ferns. Proc. Natl. Acad. Sci. USA. 91: 5730-5734.

Horikawa, Y. 1972. Atlas of Japanese Flora, an Introduction to Plant Sociology of East Asia. Gakken,

Tokyo.
Izumi, Y. & K. Ono. 1999. Changes in plastid DNA content during the life cycle of the hornwort *Anthoceros punctatus* L. Cytologia. 64: 37-44.
Kato M., Y. Yatabe, N. Sahashi & N Murakami. 2001. Taxonomic studies of *Cheiropleuria* (Dipteridaceae). Blumea. 46: 513-525.
Kim, H. N., K. Harada & T. Yamazaki. 1996. Isozyme polymorphism and genetic structure of a liverwort *Conocephalum conicum* in natural populations of Japan. Genes Genet. Syst. 76: 279-288.
Kitagawa, N. 1982. A study of *Conocephalum japonicum*, Marchantiales, Hepaticae. Acta Phytotax. Geobot. 33: 179-189 (in Japanese).
Masuyama, S., Y. Yatabe, N. Murakami & Y. Watano. 2002. Cryptic species in the fern *Ceratopteris thalictroides* (L.) Brongn. (Parkeriaceae). I. molecular analyses and crossing tests. J. Plant Res. 115: 87-97
Miwa H., J. Suhara, N. Kitagawa & N. Murakami. 2003. Biosystematic study of Japanese *Conocephalum japonicum* (Hepaticae) based on *rbcL* sequence and allozyme data. Acta Phytotax. Geobot. 54: 37-48.
Moore W. S. 1995. Inferring phylogenies from mtDNA variation-mitochondrial-gene trees versus nuclear-gene trees. Evolution. 49: 718-726.
Murakami, N., J. Yokoyama, X. Cheng, H. Iwasaki, R. Imaichi & K. Iwatsuki. 1998a. Molecular α-taxonomy of *Hymenasplenium obliquissimum* complex (Aspleniaceae) based on *rbcL* sequence comparisons. Plant Species Biol. 13: 51-56.
___, ___ & K. Iwatsuki. 1998b. *Hymenasplenium inthanonense* (Aspleniaceae), a new fern species fom Doi Inthanon, and its phylogenetic status. Thai For. Bull. 26: 40-52.
Odrzykoski, I. J. 1987. Genetic evidence for reproductive isolation between two European "forms" of *Conocephalum conicum*. Symp. Biol. Hung. 35: 577-587.
___ & J. Szweykowski. 1991. Genetic differentiation without concordant morphological divergence in the thallose liverwort *Conocephalum conicum*. Plant Syst. Evol. 178: 135-151.
Schofield W.B. & Crum H.A. 1972. Disjunctions in bryophytes. Ann. MO Bot. Gard. 59: 174-202.
Shaw A.J. 2001. Biogeographic patterns and cryptic speciation in bryophytes. J. Biogeogr. 28: 253-261.
Shiraishi, S. 1988. Inheritance of isozyme variations in Japanese black pine, *Pinus thunbergii* Partl. Silvae Genet. 37: 93-100.
Szweykowski, J., I. Odrzykoski & R. Zielinski. 1981. Further data on the geographic distribution of two genetically different forms of the liverwort *Conocephalum conicum* (L.) Dum.: the sympatric and allopatric regions. Bull. Acad. Polon. Sci., Ser. Sci. Biol. 28: 437-449.
Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin & D. G. Higgins. 1997. The CLUSTALX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24: 4876-4882.
Yatabe Y., M. Takamiya & N. Murakami. 1998. Variation in the *rbcL* sequence of *Stegnogramma pozoi* subsup. *mollisima* (Thelypteridaceae) in Japan. J. Plant Res. 111: 557-564.
___, H. Nishida & N. Murakami. 1999. Phylogeny of Osmundaceae inferred from *rbcL* nucleotide sequences and comparison to the fossil evidences. J. Plant Res. 112: 397-404.
___, S. Masuyama, D. Darnaedi & N. Murakami. 2001. Molecular systematics of the *Asplenium nidus* complex from Mt. Halimun National Park, Indonesia: evidence for reproductive isolation among three sympatric *rbcL* sequence types. Amer. J. Bot. 88: 1517-1522.
___, D. Darnaedi & N. Murakami. 2002. Allozyme analysis of cryptic species in the *Asplenium nidus* L. complex from West Java, Indonesia. J. Plant Res. 115: 483-490.

Received September 1, 2003, accepted November 19, 2003